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Comparing microbiotas in the upper aerodigestive and lower respiratory tracts of lambs

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Abstract

Background: Recently, the importance of the lung microbiota during health and disease has been examined in humans and in small animal models. Whilst sheep have been proposed as an appropriate large animal model for studying the pathophysiology of a number of important human respiratory diseases, it is clearly important to continually define the limits of agreement between these systems as new concepts emerge. In humans it has recently been established that the lung microbiota is seeded by microbes from the oral cavity. We sought to determine whether the same was true in sheep.

Results: We took lung fluid and upper aerodigestive tract (oropharyngeal) swab samples from forty lambs (seven weeks old). DNA extraction was performed and the V2-V3 region of the 16S rRNA gene was amplified by PCR then sequenced via Illumina Miseq. Oropharyngeal swabs were either dominated by bacteria commonly associated with the rumen or by bacteria commonly associated with the upper aerodigestive tract. Lung microbiota samples did not resemble either upper aerodigestive tract samples or reagent only controls. Some rumen associated bacteria were found in lung fluids, indicating that inhalation of ruminal bacteria does occur. We also identified several bacteria which were significantly more abundant in lung fluids than in upper aerodigestive tract swabs, the most predominant of which was classified as *Staphylococcus equorum*.

Conclusions: In contrast to humans, we found that that the lung microbiota of lambs is dissimilar to that of the upper aerodigestive tract and we suggest that this may be related to physiological and anatomical differences between sheep and humans. Understanding the comparative physiology and anatomy underlying differences in lung microbiota between species will provide a foundation upon which to interpret changes associated with disease and/or environment.

Keywords: lung, microbiota, sheep, lambs, oropharynx, rumen, 16S

46

47 **Background**

48 The use of 16S ribosomal RNA (rRNA) gene sequencing has facilitated the study of difficult to culture,
49 low biomass microbial communities present in the lower respiratory tract. The impact of the lung
50 microbiota on human health is a rapidly growing area of research. In order to understand this impact
51 it is important to also understand the lung microbiota dynamics during health and to include healthy
52 controls in disease studies. To achieve this, the majority of previous studies have relied on human
53 volunteers.

54 However, many individuals are hesitant to participate in research bronchoscopy due to the
55 perceived inconvenience and a fear of complications [1], despite the low risk involved. Mice and rats
56 have been used to explore the relationship between the lung microbiota and airway inflammation
57 [2-4], microbiota at different body sites [5], the environment [6], acute lung injury [7] and antibiotic
58 [8] and corticosteroid exposure [9]. However, rodents are of limited use when exploring spatial or
59 longitudinal lung microbiota dynamics due to their small lung size. Recognising the utility of large
60 animal models in this regard, and the anatomical and immunological relevance of sheep as models
61 [10-13] our group has previously used this species to explore the changes in the lung microbiota
62 upon *Pseudomonas aeruginosa* infection [14] and to explore the spatial variability present within the
63 healthy lung [15].

64 Subclinical microaspiration of pharyngeal secretions is a feature of health and this can contribute to
65 the lung microbiota composition [16] and the microbiome of the human lungs more closely
66 resembles that of the mouth than the nose or the lower gastrointestinal tract [17]. It is not yet
67 known whether the same relationship holds for species other than humans. In ruminating sheep,
68 where the oropharynx is exposed to ruminal contents on a frequent basis one would anticipate that
69 lung microbiota would similarly reflect this influence. In this paper we find that the presence of
70 rumen-like bacteria in the upper aerodigestive tract is correlated with changes in the lung

microbiota and rumen type bacteria are present in lamb lungs. We also identify bacteria which are more indicative of the lungs than the oropharynx, indicating that the presence of the sheep lung microbiota is not merely due to passive diffusion of microbes from the upper aerodigestive tract.

Methods

Animals and sampling

Scottish Mule X Suffolk lambs (20 males and 20 females, unweaned), raised on pasture from 48 hours after birth, were used in this study. These lambs were part of a study on the animal welfare implications of prenatal stress which was approved by Scotland's Rural College's (SRUC) Animal Experiments Committee and was conducted under Home Office licence. All lambs were raised by their dams and prior to euthanasia their only food sources were ewe's milk and pasture. At seven weeks old (mean age = 48.8 days \pm 0.8 standard deviation (SD); mean weight \pm SD = 20.6 kg \pm 2.6 kg), lambs were euthanized by barbiturate overdose then the cadavers were transported from the farm to the dissecting room (~5 min). Oropharyngeal swabs were taken using cotton tipped swabs (Swab Plain Wood Cotton Tip Sterile (710-0181), Copan, Italy). To prevent oral contamination, swabs were stored in protective plastic sheaths from which the swab could be advanced and retracted once it was positioned at the sampling site. Swabs were then transferred into a new plastic sheath and stored on ice.

The ventral aspect of the neck was shaved and a sterile scalpel used to incise through the skin and subcutaneous tissues to expose the ventral surface of the trachea. A sampling site was identified on the exposed ventral surface and the trachea cranial to this site was completely closed off by both string ligature and clamp placement. The selected sampling site was then heat seared and 50 ml of sterile phosphate buffered saline (PBS) was injected through the seared section into the tracheal lumen. The head and neck were oriented such that the PBS would flow caudally down the thorax. A

second clamp was immediately placed caudal to the site of injection to prevent backflow, leakage and potential contamination. The lamb cadavers were then tipped so that the PBS would run caudally into their lungs and then tipped back again so that the fluid would collect in the tracheal lumen immediately caudal to the position of the second clamp. A sampling site identified on the ventral surface of the trachea was seared and a needle and syringe were used to collect the pooled fluid. On average 4 ± 1.7 ml (mean \pm SD) of lung fluid was collected per animal. Lung fluid was stored on ice until further processing. Oropharyngeal swabs were sterilely cut into 500 μ l PBS. Lung fluids were centrifuged at 13,000 g for 5 min. The supernatant was removed and the pellets were resuspended in 500 μ l PBS. Oropharyngeal swabs and lung fluids were stored at -80°C until DNA extraction.

DNA extraction, amplification, and sequencing

DNA extractions using the PowerSoil DNA isolation Kit (Mo Bio, Carlsbad, USA) and quantitative PCR (qPCR) using the 16S rRNA gene qPCR primers UniF340 (5-ACTCCTACGGGAGGCAGCAGT-3) and UniR514 (5-ATTACCGCGGCTGCTGGC-3) were performed as described previously [15]. Extraction kit reagent controls, consisting of reagent only extractions, were produced for every day DNA extractions were performed. PBS controls were created by extracting DNA from 500 μ l of the PBS which had also been used to wash out the lamb lungs. A mock community control was included which has been described previously [15].

A nested PCR reaction was used to produce amplicons for sequencing; this technique was chosen to reduce PCR bias caused by barcoded primers [18]. The first round of PCR amplified the V1-V4 16S hypervariable regions using the primers 28F (5-GAGTTTGATCNTGGCTCAG-3) and 805R (5-GACTACCAGGGTATCTAATC-3). The conditions were: 94°C for 2 min followed by 20 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1.5 min followed by a final extension step of 72°C for 20 min. Clean-up was performed using the AMPure XP PCR purification system (Beckman Coulter, Brea, USA).

In a previous study, we found that PCR bias in high template samples could be reduced by diluting amplicons from the first round of PCR to a similar concentration to those of lung fluid samples [15]. Therefore, in this study we used our qPCR values to calculate the dilutions needed to achieve this. The second round of PCR used the barcoded V2-V3 primers 104F (5-GGCGVACGGGTGAGTAA-3) and 519R (5-GTNTTACNGCGGCKGCTG-3). The dilutions and barcoded primers used for each sample can be found in **Additional file 1**. The PCR conditions were: 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 67°C for 30 s, and 72°C for 10 s followed by a final extension step of 72°C for 2 min. The amplicons were again purified using the AMPure XP PCR purification system.

Bioinformatic and statistical analysis

Samples were sequenced via either Illumina Miseq or Hiseq runs (Illumina, San Diego, USA) (**Additional file 1**) producing 250 base pair paired-end reads. Cutadapt [19] was used to remove primers. Quality control and taxonomic assignment of sequences was carried out within mothur [20] following a protocol created by the mothur developers [21], adjusted to suit our dataset [15]. Sequences were subsampled before statistical analysis. The sequencing error rate, principal coordinate analysis graphs (PCOA); analyses of molecular variance (AMOVA); Good's coverage analyses [22]; richness (Chao 1 Index) and diversity (Inverse Simpson Index) calculations and indicator analyses [23] were all calculated within mothur. Clustering of microbial communities into metacommunities was also carried out within mothur using a probabilistic modelling technique based upon work by Holmes *et al* [24]. The significance of differences between the diversity and richness of groups was calculated using either the two sample t-test (normal data) or the Mann-Whitney U test (non-normal data) within Minitab 16 for Windows (Minitab, Coventry, UK). Heatmaps were constructed in R Version 3.2.2 [25] using the Vegan [26], RColorBrewer [27], gplots [28] and heatplus [29] packages. Clustering within heatmaps was performed using the Bray-Curtis dissimilarity [30]. Sequences can be accessed via the Bioproject accession number PRJNA317719.

Results

Quality assurance of methodology

11,878,769 sequence reads were produced in total with an average of $138,125 \pm 29,306$ per sample (mean \pm SD). The sequencing error rate was calculated as 0.35%. The oropharyngeal swab sample from lamb 12773 was found to have very low read numbers and was therefore discarded from statistical analyses, as was its corresponding lung fluid sample. A total of 1061 OTUs were identified (**Additional file 2**) which were reduced to 750 after sub-sampling. All Good's coverage values were > 0.999 indicating that at least 99.9% of the bacteria present in our samples are likely to have been identified. The most abundant bacterial OTUs from extraction kit reagent only controls are listed in **Table 1**. The similarity of the OTUs found on the 25th and 26th March 2015 is likely due to the fact that the same lot of extraction kit was used. Upon examining our data we found that lung fluid samples clustered by when they were processed (**Additional file 3**). Samples sequenced via Miseq and Hiseq underwent DNA extraction and PCR amplification separately. We identified two OTUs which were significantly indicative ($P < 0.05$) of samples from either the Hiseq or Miseq run which were also present in all lung fluid samples from the run they were indicative of: OTU 4 (*Pseudomonas*) and OTU 112 (Yaniellaceae). These OTUs are likely due to contamination and were therefore removed prior to analysis.

Table 1: Bacterial OTUs found to be $> 5\%$ abundant in extraction kit reagent controls

Date of DNA extraction	OTUs	Abundance
17th July 2014	<i>Aerococcus</i>	14%
	Dermabacteraceae	12%
	<i>Micrococcus</i>	10%

	<i>Enhydrobacter</i>	9%
	<i>Leuconostoc</i>	6%
	<i>Kocuria</i>	6%
	<i>Actinomyces</i>	6%
25 th March 2015	<i>Methylobacterium komagatae</i>	65%
	Ruminococcaceae	11%
	<i>Methylobacterium</i>	6%
26 th March 2015	<i>Methylobacterium komagatae</i>	67%
	<i>Methylobacterium</i>	6%

165

166

167 **Lamb oropharyngeal swabs cluster into two distinct community types**

168 Oropharyngeal swabs were taken from 40 lambs. Using the Laplace approximation it was found that

169 swabs could be partitioned into two separate groups based upon the types of bacteria present.

170 These appeared to correspond to either oropharyngeal-type (partition 1) or rumen-type (partition 2)

171 bacteria (**Additional file 4**). Oropharyngeal-type communities were dominated by the OTUs

172 Pasteurellaceae (22%), *Mannheimia* (14%), *Fusobacterium* (11%), *Bibersteinia trehalosi* (8%),

173 Neisseriaceae (7%), *Moraxella* (6%) and *Bibersteinia* (5%). Rumen-type communities were

174 dominated by the OTUs *Prevotella* (36%), Clostridiales (11%), Ruminococcaceae (7%),

175 Lachnospiraceae (6%) and *Butyrivibrio* (6%).

176 The richness (chao: non-normal data) and diversity (Inverse Simpsons: normal data) of the partitions

177 were compared. There was no significant difference in richness or diversity between the rumen-type

178 partition and the oropharyngeal-type partition.

179

180 **Dichotomous oropharyngeal microbiota are associated with different lung community structures**

181 The most common OTUs found in lung fluid samples were *Staphylococcus equorum* (13%),
182 *Staphylococcus sciuri* (6%), *Mannheimia* (5%) and *Prevotella* (5%). Using the Laplace approximation,
183 lung fluids did not cluster into more than one group. Lung fluids were then manually partitioned into
184 the same groups as swabs. A significant difference in bacterial community structure was found
185 between these groups (AMOVA: $P = 0.016$) and a small number of OTUs were found to be
186 significantly different between the two groups. *Prevotella* ($P = 0.03$) and *Sphingobium* ($P = 0.039$)
187 were significantly indicative of lambs from which rumen-type swabs were derived whereas
188 *Paracoccus aminovorans* ($P = 0.036$) was indicative of lambs from which oropharyngeal-type swabs
189 were derived. **Figures 1 and 2** contain visual representations of sample clustering.

190 We compared the proportions of the dominant OTUs in rumen-type swabs with their corresponding
191 proportions in lung samples. On average, these OTUs were found in the following proportions in lung
192 samples: *Prevotella* (5%), Clostridiales (2%), Ruminococcaceae (3%), Lachnospiraceae (1%) and
193 *Butyrivibrio* (1%).

194

195 **The presence of a lung specific microbiota**

196 Indicator species analysis determined that several OTUs were significantly more indicative of the
197 lungs than of oropharyngeal swabs (**Table 2**). It is likely that reagent contamination will have had
198 more of an impact on the lung fluid samples than on the oropharyngeal swabs, due to their lower
199 biomass. However, when examining the indicative OTUs, the majority of samples were not found to
200 contain the same proportions of these OTUs as the PBS controls processed alongside them (**Figure**
201 **3**). Of the indicative OTUs, by far the most common was *Staphylococcus equorum* which constituted,

on average, 13.3% of the total bacteria present in lung fluids and which was only present in low numbers in controls and oropharyngeal swabs.

Table 2: OTUs significantly more indicative of lung fluids than oropharyngeal swabs.

Taxonomy	P value	Average proportion in lung fluids (Mean \pm SD)	Average proportion in oropharyngeal swabs (Mean \pm SD)	Highest proportion in PBS controls
<i>Brachybacterium</i>	0.006	1.0% \pm 1.7%	0.035% \pm 0.10%	0.022%
<i>Brevibacterium</i>	0.002	1.2% \pm 1.4%	0.064% \pm 0.24%	0%
<i>Corynebacterium</i>	< 0.001	1.9% \pm 2.3%	0.065% \pm 0.19%	0.044%
<i>Delftia</i>	< 0.001	0.80% \pm 1.7%	0% \pm 0%	0%
Enterobacteriaceae	0.023	0.65% \pm 2.6%	0.0063% \pm 0.029%	2.2%
<i>Frigoribacterium</i>	0.021	0.79% \pm 1.4%	0.077% \pm 0.31%	0%
<i>Janthinobacterium</i>	0.01	0.57% \pm 1.4%	0.0023% \pm 0.0068%	0%
<i>Jeotgalicoccus psychrophilus</i>	0.008	1.6% \pm 2.1%	0.040% \pm 0.10%	0%
<i>Microbacterium aurum</i>	0.047	1.2% \pm 2.8%	0.0045% \pm 0.013%	0%
<i>Micrococcus</i>	0.017	0.77% \pm 1.6%	0.0080% \pm 0.029%	4.4%
Oxalobacteraceae	< 0.001	0.96% \pm 1.5%	0.012% \pm 0.043%	3.0%
<i>Pelomonas</i>	< 0.001	0.65% \pm 1.1%	0.00057% \pm 0.0036%	2.0%
Peptostreptococcaceae	0.006	1.8% \pm 2.2%	0.050% \pm 0.11%	0.044%
<i>Propionibacterium acnes</i>	< 0.001	0.84% \pm 2.3%	0.0040% \pm 0.020%	1.6%
<i>Pseudomonas citronellolis</i>	< 0.001	0.51% \pm 1.1%	0% \pm 0%	1.0%
<i>Rathayibacter caricis</i>	0.016	0.58% \pm 1.2%	0.0057% \pm 0.021%	0%
<i>Saccharopolyspora</i>	0.009	0.52% \pm 1.2%	0.0011% \pm 0.0071%	0%
<i>SMB53</i>	< 0.001	0.71% \pm 1.3%	0.0045% \pm 0.018%	0%

<i>Sphingobium yanoikuyae</i>	< 0.001	0.53% ± 0.56%	0% ± 0%	13%
<i>Staphylococcus</i>	< 0.001	3.9% ± 5.4%	0.060% ± 0.18%	8.6%
<i>Staphylococcus equorum</i>	< 0.001	13.3% ± 9.6%	0.32% ± 0.97%	0.044%
<i>Staphylococcus sciuri</i>	< 0.001	6.4% ± 5.3%	0.18% ± 0.59%	2.0%
<i>Streptomyces</i>	< 0.001	2.0% ± 3.8%	0.025% ± 0.096%	0%
<i>Turicibacter</i>	0.016	1.0% ± 1.8%	0.0074% ± 0.021%	0%
<i>Variovorax paradoxus</i>	0.011	1.2% ± 1.2%	0.024% ± 0.060%	0.022%

OTUs which were significantly more (P < 0.05) indicative of lamb lung fluids than oropharyngeal swabs and which were on average > 0.5% abundant in lung fluids.

Discussion

Sheep are commonly used as large animals models of the respiratory system due to the physiological and immunological similarities of their lungs to those of humans [11, 10, 31, 32]. We have previously used the sheep to study both the extent of variation in the lung microbiota [15] and the direct and remote changes in the lung microbiota caused by localised *P.aeruginosa* infection and antibiotic treatment [14]. As the sheep is an important agricultural animal, studies of their respiratory microbial communities may also be of interest from an animal health perspective.

It has previously been demonstrated that microaspiration of microbes from the upper aerodigestive tract is common in humans and can lead to an inflammatory phenotype [33]. When microbial communities from healthy human lungs are characterised they are often found to contain microbes associated with the upper aerodigestive tract [34]. The healthy human lung microbiota is thought to be formed predominantly from the neutral dispersal of these upper aerodigestive tract microbes into the lungs rather than by the differential growth of lung adapted microbial communities [35]. We sought to identify whether this was also the case in sheep.

223 Sheep oropharyngeal swabs could be partitioned into two separate groups which were
224 predominantly composed of OTUs identified as bacteria which are well known members of either
225 the rumen (*Prevotella*, Clostridiales, Ruminococcaceae, Lachnospiraceae and *Butyrivibrio* [36-38]) or
226 respiratory tract microbiotas (Pasteurellaceae, *Mannheimia*, *Fusobacterium*, *Bibersteinia trehalosi*,
227 Neisseriaceae, *Moraxella* and *Bibersteinia* [39-41]). These bacteria were also detected in a previous
228 study examining sheep buccal swabs [42].

229 It is not possible to identify whether this dichotomy reflected recent rumination, or some stochastic
230 post-mortem leakage of rumen fluid into the oropharynx in some individuals. The lambs during this
231 study were not weaned but were at an age when it is expected that they all would be regularly
232 supplementing their diet with grass and would be ruminating.

233 Regardless of the drivers of this oropharyngeal dichotomy, the microbial communities found in the
234 lungs were very different to those found in both the rumen- and oropharyngeal-type swabs. A large
235 number of bacterial OTUs were found to be significantly more abundant in lung fluids in comparison
236 to oropharyngeal swabs, including *Staphylococcus equorum* which was by far the most common
237 bacterial OTU found. Several OTUs which are commonly associated with the rumen were also
238 identified in lung fluids. Our lung fluid samples will have been more affected by reagent
239 contamination than the oropharyngeal swabs due to the lower quantity of bacterial DNA present
240 [43]. However, the microbial communities found in lung fluids did not reflect the bacteria found in
241 reagent only controls processed on the same day so the presence of bacteria in the lamb lung
242 cannot be attributed purely to sample contamination. Nor can it be attributed to disease as no
243 lambs showed clinically overt signs of respiratory illness during the study.

244 There are several reasons why the microbes found in lamb lungs might not reflect those found in the
245 upper aerodigestive tract to the same extent as is found in humans. Sheep have evolved to cope
246 with rumination and thereby may have more efficient anatomical barriers to microaspiration [44].

Physiological and anatomical differences such as the horizontal disposition of the lungs, increased nasal breathing and increased saliva production [45, 46] may also contribute.

Conclusions

In this study we examined oropharyngeal swab and lung fluid samples taken from healthy lambs to characterise the bacterial communities present and to assess the impact of rumination on these communities. We found that oropharyngeal swabs were dominated by either rumen-type or oropharyngeal-type microbial communities. We also found that lung bacteria did not greatly resemble either rumen- or oropharyngeal-type swabs and identified several bacterial OTUs which were more indicative of lung fluids. The lungs did contain several rumen associated bacteria which may indicate that there is a certain degree of microaspiration of ruminal contents in lambs.

Sheep are not human but the opportunities that they, and other large animals, present offer valuable insights into the dynamic relationship of the upper aerodigestive and lower respiratory tract microbiota in health. In the future, their value may extend to developing an understanding of the factors that predispose the upper aerodigestive tract microbiota towards disease in the lower respiratory tract.

Declarations

Ethics approval and consent to participate

Animals were used which were part of a study examining the effects of prenatal stress. This study was approved by Scotland's Rural College's (SRUC) Animal Experiments Committee and was conducted under Home Office licence.

270

271 **Consent for publication**

272 Not applicable

273

274 **Availability of data and materials**

275 The datasets generated during and/or analysed during the current study are available in the Short

276 Read Archive and can be accessed at Bioproject accession number PRJNA317719

277 (<http://www.ncbi.nlm.nih.gov/bioproject/PRJNA317719>).

278

279 **Competing interests**

280 The authors declare that they have no competing interests.

281

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288 or the writing of this manuscript.

289

290 **Authors' contributions**

LG contributed to study design, drafting of paper and sample collection, processing and analysis. DC contributed to study design, drafting of paper and sample collection. SW contributed to drafting of paper and sample collection. KR contributed to study design and the drafting of paper. GM contributed to study design, drafting of paper and sample collection. All authors read and approved the final manuscript.

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Figure Legends

Figure 1: PCOA graph showing the relatedness of upper aerodigestive tract samples from lambs partitioned into 2 groups using the Laplace approximation. Lung fluids belonging to the same animals were partitioned into the same groups. Lung fluid partitions clustered significantly separately by AMOVA ($P = 0.016$) as did oropharyngeal swabs ($P < 0.001$). Controls are PBS and extraction kit reagent controls.

Figure 2: Heatmap of OTUs found in lamb lung fluids, oropharyngeal swabs, extraction kit reagent and PBS controls. OTUs were included when they were $> 5\%$ abundant in at least one sample. Oropharyngeal swabs partitioned into rumen-like bacterial communities are indicated by green whereas those which were upper aerodigestive tract-like are indicated by a red line. The lung fluid samples from the oropharyngeal-like animals are indicated by blue whereas those from the rumen-type animals are indicated by pink.

Figure 3: Heatmap of bacterial OTUs found to be more indicative of lamb lung fluids than oropharyngeal swabs ($P < 0.05$). Fluid and PBS samples from which DNA was extracted on specific

440 dates are surrounded by coloured lines: 17th July 2014 (blue) and 25th March 2015 (red). DNA
441 extractions carried out on 26th March 2015 only comprised oropharyngeal swabs which are not
442 included in this figure.

443

444 **Additional files**

445 Additional file 1 (Additional file 1.xlsx): **Dataset S1**: Sample processing data for all samples.

446 Additional file 2 (Additional file 2.xlsx): **Dataset S2**: Full list of bacterial OTUs and taxonomies.

447 Additional file 3 (Additional file 3.docx): **Figure S1**: Heatmap of OTUs found in lamb lung fluids,
448 oropharyngeal swabs, PBS and extraction kit reagent only controls.

449 Additional file 4 (Additional file 4.docx): **Table S1**: OTUs responsible for partitioning of lamb
450 oropharyngeal swabs into two groups (using Laplace value).